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Influence of different inductors and operating conditions in the production of lipase from *Aspergillus niger* using cassava peel: a short study

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Abstract: The lipase production using microorganisms is very advantageous, especially because of their high productivity and the possibility of use of agro-industrial wastes as substrate. In the present study, there was the possibility that supplementation of cassava peel with different inductors (soybean oil, maize oil, frying oil, swine fat, glucose and yeast extract). Subsequently it was sought to maximize the production of the enzyme by varying the concentration of inductor and the fermentation temperature using the technique of experimental design and response surface. In the first step of the study, were obtained as better inductor swine fat, compared to vegetable oils and other inductors. The swine fat used as inductor resulted a lipase activity of 70.82 ± 6.07 U/g, this value being statistically equal ($p>0.05$) to values of lipase activity obtained from the cassava peel supplemented with soybean oil (2.5% w/w) + glucose (1% w/w) and maize oil (2.5% w/w) + glucose (1% w/w). In optimizing of temperature and concentration of inductor, was defined, from the statistical treatments, 36°C as the optimum temperature for fermentation and 1% (w/w) as the concentration of inductor (swine fat), with maximum activity lipase obtained from 72.26 ± 2.56 U/g.

Keywords: Cassava peel, inductors, solid state fermentation, operational variables.

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1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are important industrial enzymes due to their versatile applications (Salihu *et al.*, 2012). They need to be robust and versatile with respect to the range of substrates they can act on, but at the same time they should have a high specificity for the reactions they catalyze (Gupta *et al.*, 2004).

Lipases catalyze the hydrolysis of acyl glycerol to fatty acids, di-acyl glycerol, mono-acyl glycerol and glycerol. Under certain conditions, they also catalyze the synthesis of esters by transesterification, thioesterification and aminolysis (Mahadik *et al.* 2002).

Microbial lipases have considerable industrial potential as a catalyst for hydrolysis, synthesis and trans-esterification of tri-acyglycerol owing to advantages such as high levels of production and diversity of stero-specific properties (Tan *et al.*, 2003). The unique properties of lipase catalyzed reactions such as chemoselectivity, regioselectivity, stereoselectivity, non-requirement of cofactors and stability in organic solvents make microbial lipase a versatile biocatalyst in many industrial applications such as foods, cosmetics, detergents, biosensors, pharmaceuticals, and recently in the field of bioenergy especially in biodiesel production (Salihu *et al.*, 2011).

The natural substrates of lipases are practically insoluble in water, so the reaction is catalyzed at the water–lipid interface and does not follow the classic Michaelis–Menten kinetics (Guncheva and Zhiryakova, 2011).

In the last two decades, the Solid-State Fermentation has attracted increasing attention for the production of enzymes, metabolites, etc., due to several biotechnological advantages such as higher fermentation productivity, higher end-product concentration, higher product stability and lower catabolic repression (Li and Zong 2010).

The Solid-State Fermentation (SSF) is defined as the fermentation on moist solid substrate in the absence or near absence of free water, thus being close to the natural environment to which microorganisms are adapted (Li and Zong 2010; Santis-Navarro *et al.*, 2011), mainly filamentous fungi as *Aspergillus niger*.

Filamentous fungi are recognized as the best lipase producers and are currently the preferred sources since they produce extracellular lipases, facilitating the extraction from fermentation media (Contesini *et al.*, 2010).

Several factors have been reported to affect the extracellular lipase production such as pH, temperature, aeration and medium composition (Salihu *et al.*, 2011). Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production. These requirements are influenced by the type and concentration of the carbon and nitrogen sources, culture pH and growth temperature (Contesini *et al.*, 2010).

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Therefore, the improvement in productivity of lipases can be achieved through manipulation of nutritional as well as physical parameters (Salihu *et al.*, 2011). Lipidic carbon sources generally seem to be essential to obtain a high lipase yield, although a few authors observed that the presence of fats and oils was not statistically significant for enzyme production (Contesini *et al.*, 2010).

Therefore, the objective of this study was to produce lipase from *Aspergillus niger* in SSF using cassava peel added with different inducers. Subsequently, verify the operating conditions (temperature and concentration of inducer) for maximizing the production of the enzyme.

2. Material and methods

The experiments for the production of lipase were conducted at the Laboratory of Bioprocess and Laboratory of Microbiology, Department of Food Engineering and Chemical Engineering (DEAQ), State University of Santa Catarina (UDESC).

2.1 Microbial culture and inoculum preparation

The propagation of spores of *Aspergillus niger* prior to fermentation was carried out for 7 days at 27.5ºC in a medium constituted by potato dextrose agar (PDA) 3.9% (w/v) and distilled water. Medium for inoculum production consisted of (w/v): 2% starch, 1% olive oil, 0.1% yeast extract, 0.025% $MgSO_4.7H_2O$, 0.05% KH_2PO_4, 0.5% CaCO3 and 1.5% agar. This medium was inoculated with a spore suspension and incubated at 27ºC for 7 days. The spores were collected adding 10mL of a sterile 0.1% Tween 80 aqueous solution and glass beads to the fermented agar medium and kept at 4ºC until use (Kempka *et al*., 2008).

2.2 Physicochemical characterization of cassava peel

The physicochemical characterizations of cassava peel, such as pH, moisture, ash and starch were analyzed. For the determination of pH, 5g of peel homogenized in 5mL distilled water were used and preceded with the reading digital potentiometer (Quimis).

For the moisture determination, the authors used the gravimetric method. For the determination of ash, incineration was used in a muffle at 550°C and for determination of starch, the authors used the method of acid digestion and determination of sugars reducers (Adolfo Lutz Institute, 2008).

2.3 Preparation of the medium of solid state fermentation

Was used as substrate (cassava peel) previously comminuted and dried in an oven temperature of 105°C for 24 hours and subsequently frozen for maintaining their physicochemical

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characteristics. The experiments for lipase production were carried out aseptically in conical reactors covered with hydrophobic fabric. Aqueous solution containing the inductors was added to the substrate and the resulting medium was then sterilized at 121ºC for 20 minutes.

The sterile medium was then inoculated with 108 spores/g dry substrate using spore suspension previously prepared. Cultivation was carried out in an incubator BOD during 60 hours according to Gerber et al. (2013). The moisture of the medium was set at 55% in accordance with Kempka et al., (2008).

2.4 Study of inductors to the production of lipase

The inductors used in this study were soybean oil, maize oil, frying oil (collected in a restaurant after being reused), swine fat (oils and fats was considered a supplementary carbon source since their composition is mainly constituted by lipids), glucose and yeast extract. Table 1 shows the experiments with inductors used and the concentration of each inductor.

2.5 Optimization of lipase production

After defining the inductor, held a factorial design with 2 factors and 4 axial points (22) out to evaluate the effect of incubation temperature (22.7–38.2ºC) and inductor concentration (0.4–4.6%) on lipase production. The range of the factors investigated was selected based on previous work (Gerber *et al*., 2013). Each assay was carried out in duplicate.

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Table 1. Inductors used in the production of lipase from *Aspergillus niger* using cassava peel as substrate.

Experiment	Inductors (%, m/m)
SO	Soybean oil (2.5 %)
MO	Maize oil (2.5 %)
FO	Frying oil (2.5 %)
SF	Swine fat (2.5 %)
SO+G	Soybean oil (2.5 %) + Glucose (1 %)
MO+G	Maize oil (2.5 %) + Glucose (1 %)
FO+G	Frying oil (2.5 %) + Glucose (1 %)
SF+G	Swine fat (2.5 %) + Glucose (1 %)
SO+G+YE	Soybean oil (2.5 %) + Glucose (1 %) + Yeast extract (1 %)
MO+G+YE	Maize oil (2.5 %) + Glucose (1 %) + Yeast extract (1 %)
FO+G+YE	Frying oil (2.5 %) + Glucose (1 %) + Yeast extract (1 %)
SF+G+YE	Swine fat (2.5 %) + Glucose (1 %) + Yeast extract (1 %)

2.6 Extraction and determination of lipase activity

The fermented medium was weighed, added to 45mL of 0.1 mol.L-1 sodium phosphate buffer at pH 7.0 and incubated at 35ºC and 200 rpm for 30 minutes for enzyme extraction. Following extraction, the liquid fraction was separated by filtration and assayed for lipase activities.

Lipase activity was determined by titration method. An emulsion of olive oil (10% w/v) and arabic gum (5% w/v) in sodium phosphate buffer 0.1mol.L-1, pH 7.0 was incubated with a sample of the enzymatic extract at 37ºC and 160 rpm for 15 minutes. The reaction was stopped and the fat acids extracted with a solution of acetone and ethanol (1:1). The fatty acids produced were titrated with NaOH 0.05 mol.L-1 (Freire *et al*., 1997). One unit of lipase activity was defined as the amount of enzyme that produces 1 µmol of fatty acids/minutes, under the assay conditions.

2.7 Statistical analysis

Data was analyzed using the Statistica® 10.0 (Statsoft Inc.). Significant differences ($p<0.05$) between means were identified using Tukey test.

3. Results and discussion

In physicochemical characterization of cassava peel was obtained as humidity value before drying 72.26%, ash 2.27%, pH 6.0 and 56.5% starch, proving to be an appropriate substrate for fermentation. The lipase production was possible in this way due to the supplementation of inductors.

3.1 Supplementation effect of inductors in lipase activity

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Table 2 shows the results of enzyme activity for the experiments with different inductors.

Table 2. Means of results of lipase activity for lipase production using cassava peel and different inductors.

Experiment	Lipase activity (U/g)
SO	$26{,}24^{a} \pm 6{,}24$
MO	$22.16^{a} \pm 2.59$
FO	$23.38^{a} \pm 2.36$
SF	$70.82^{cd} \pm 6.07$
SO+G	$86.38^{d} \pm 0.48$
MO+G	$74.38^{cd} \pm 5.38$
FO+G	$59.61^{bc} \pm 7.39$
SF+G	$62.54^{bc} \pm 6.14$
SO+G+YE	$52.62^{b} \pm 5.52$
MO+G+YE	$59.75^{bc} \pm 8.50$
FO+G+YE	$61.20^{bc} \pm 4.96$
SF+G+YE	$60.92^{bc} \pm 9.88$

Mean lipase activity followed by different letters differ significantly with 95% confidence ($p<0.05$) by Tukey test.

It is found that the highest values of lipase activity were obtained for the experiments with the addition of soybean oil and glucose (SO+G), with addition of maize oil and glucose (MO+G) and with addition of swine fat (SF), statistically equal ($p>0.05$) between themselves and different ($p<0.05$) from other experiments, by Tukey test.

Colla et al. (2010) who studied the lipase production using soybean and rice husk obtained 22.5 U/g of lipase activity. Contesini et al. (2009) obtained 33.03 U/g of lipase activity using wheat bran as substrate. In the two studies cited, the micro-organisms used were Aspergillus.

The experiments with lower values of lipase activity (consequent decreased production of the enzyme) were experiments with the addition of soybean oil (SO), maize oil (MO) and frying oil (FO), statistically equivalent ($p>0.05$), and none of these experiments was supplemented with glucose or yeast extract.

The production of lipase is mostly inducer dependent; in many cases, oils act as good inducers of the enzyme. The requirement of sugar as a carbon source in addition to lipids varies with the microorganism. In general, media supplemented with glucose along with triglycerides stimulate maximum lipase production in the case of fungi (Rigo *et al*., 2010). The swine fat is rich in palmitic and oleic acids in the approximate ratio of 1:2, corresponding to about 75% of its fatty acids (Silva and Gioielli, 2006).

Ramani *et al*. (2010) using lipase from Pseudomonas gessardii showed the highest percentage of hydrolysis using as substrate the bovine fat (146%) followed by goat fat (112%) and olive oil (100%). Affirms that the capacity of lipase to hydrolyze all substrates has great potential for application in the hydrolysis of lipids for the treatment of wastewater.

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For the next steps, it was decided to as an inducer the fat swine, for being an economically viable alternative as inductor in relation other inductors that showed similar results statistically.

3.2 Optimization of lipase production

Table 3 presents the matrix of the complete factorial design with the lipase activity obtained at 60 hours of fermentation. The highest lipase production, showed higher amount of lipase activity occurred to the experiment E3, with mean 72.26 U/g lipase activity of, with a temperature of 36°C and the percentage of inductor 1.0 (w/w). By Tukey test can verify that the result of the experiment E3 differs significantly ($p<0.05$) for all other experiments.

Table 3. Matrix of the experimental design (real and coded values) with responses in terms of lipase activity for lipase production using swine fat as inductor.

Experiment	Temperature (ºC)	Inductor concentration (%, m/m)	Lipase activity (U/g)*
E1	25 (-1)	1.0 (-1)	$18.15^{b} \pm 0.21$
E2	25 (-1)	4.0 (+1)	$34.22^{c} \pm 1.67$
E3	36 (+1)	1.0 (-1)	$72.26^{g} \pm 2.55$
E4	36 (+1)	4.0 (+1)	$67.56^{f} \pm 3.35$
E5	22.7 (-1.41)	2.5 (0)	$10.93^{a} \pm 3.03$
E6	38.2 (+1.41)	2.5 (0)	$52.63^{e} \pm 6.56$
E7	30.5 (0)	0.4 (-1.41)	$43.99^{d} \pm 0.66$
E8	30.5 (0)	4.6 (+1.41)	$47.03^{d} \pm 0.46$
E9 (C)	30.5 (0)	2.5 (0)	$48.33^{d} \pm 1.13$
E10 (C)	30.5 (0)	2.5 (0)	$50.46^{de} \pm 2.64$

Mean lipase activity followed by different letters differ significantly with 95% confidence ($p<0.05$) by Tukey test. * Lipase activity ± Standard deviation (±)

The enzyme productivity was also determined in 60 hours of fermentation being achieved 1.20 U/g.h (experiment E3). This value is higher that obtained by Kempka *et al*. (2008), 0.99 U/g.h in 60 hours of fermentation using *Aspergillus niger*, by Gerber *et al*. (2013), 0.74 U/g.h in 72 hours of fermentation using *Penicillium verrucosum*, by Gutarra *et al*. (2005), 0.26 U/g.h in 72 hours of fermentation and by Mahadik *et al*. (2002), 0.87 U/g.h in 144 hours of fermentation using *Aspergillus niger* and wheat bran/sunflower oil as substrate.

Biological processes are highly complex and the enzyme production depends on the interaction of several processes influencing microbial cellular metabolism (Gerber *et al*., 2013). Figure 1 shows a Pareto Diagram obtained from experimental results of the lipase activity.

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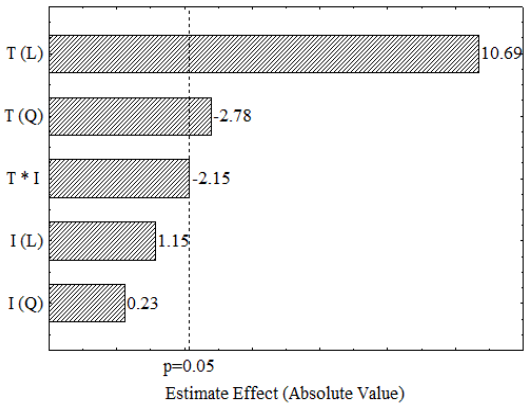

Figure 1. Pareto chart obtained from experimental results of the lipase activity using cassava peel as substrate and swine fat as inductor.

$$LA = 296.08 + 17.62 * T - 0.21 * T^2 - 0.63 * T * I \quad \text{(Eq.1)}$$

where LA denotes lipase activity (U/g dry substrate), T is the temperature and I the inductor concentration.

The model generated a response surface for lipase activity (R2=0.904), shown in Figure 2. This surface indicates that the conditions that maximize lipase activity production are 36ºC, any of inductor concentration (swine fat) within the range tested.

The experiments where there was a higher lipase activity were E3 (36ºC and 1% of swine fat), E4 (36ºC and 4.0% of swine fat) and E6 (38.2ºC and 2.5% of swine fat). Therefore, the condition that presents the greatest advantage is the corresponding experiment E3, that among the listed above, has the lowest temperature and lower concentration of inductor.

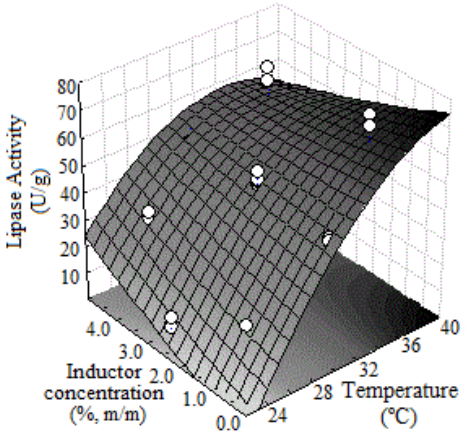

Figure 2. Response surface for lipase activity as a function of inductor concentration and temperature.

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4. Conclusions

The fungus *Aspergillus niger* showed a good performance in the enzyme production. The best inductor for the lipase production was swine fat which provided a lipase activity of 70.82 U/g (T=27.5 ° C, 2.5% w/w of an inductor). In relation the variables for optimization of lipase production using fat swine as inductor (the inductor temperature and percentage), the temperature had a positive significant effect on lipase activity. The temperature of 36°C and a concentration of 1% (w/w) fat swine led to the maximum lipase activity, as a value of 72.26 U/g. More studies are needed to complement the present study.

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